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Calculating DNA yield from different direct lysis procedures on blood deposited onto various fabrics

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Thesis

CALCULATING DNA YIELD FROM DIFFERENT DIRECT LYSIS PROCEDURES ON BLOOD DEPOSITED ONTO VARIOUS FABRICS

by

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ABSTRACT

The goal of forensic DNA analysis is to develop a profile from an item of evidence to create linkages in a crime. To generate an interpretable short tandem repeat (STR) profile, purified Deoxyribonucleic Acid (DNA) must be extracted from the cell.

Silica-binding extractions consist of several purification steps that yield Polymerase Chain Reaction (PCR)-ready DNA in the solution. However, these purification steps introduce the potential for loss of DNA. Direct lysis methods eliminate the potential for loss of DNA since extraction can be done in one tube. Conversely, since direct lysis methods do not contain a purification step, the resulting extracted DNA lysate may contain inhibitors that prevent successful PCR analysis.

Samples retrieved from crime scenes are typically poor or degraded. Blood is a body fluid commonly encountered at crime scenes or on items of evidence, however; it contains many PCR inhibitors that affect downstream DNA analysis.

The purpose of this study is to evaluate DNA recovery from whole blood deposited on cotton, nylon, wool, rayon, or cotton swabs using several available direct lysis reagents.

Evaluation and comparison of four direct lysis reagents are reported.

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LIST OF ABBREVIATIONS

CE	Capillary Electrophoresis
cm	Centimeters
°C	Degrees Celsius
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
IPC	Internal Positive Control
ISO	International Organization for Standardization
LCM	Laser Capture Microdissection
Y_{10}	Luminance
μL	Microliters
mL	Milliliters
mm	Millimeter
mM	Millimolar
ng	Nanograms
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
qPCR	Quantitative Polymerase Chain Reaction
RCF	Relative Centrifugal Force
RFU	Relative Fluorescence Units
SDS	Sodium Dodecyl Sulfate
STR	Short Tandem Repeat

T_{10}	Tint
C_t	Threshold Cycle
TE	Tris-EDTA
W_{10}	Whiteness

1. INTRODUCTION

1.1 Forensic Applications of DNA Analysis

Deoxyribonucleic Acid (DNA) is unique to every individual (1). Thus, the goal of forensic DNA analysis is to obtain a profile from an unknown evidence item for the purpose of identifying a contributor to create linkages between evidence, crime scene(s), suspect(s), and victim(s) (1,2). This is most commonly done using Polymerase Chain Reaction (PCR)-based amplification of highly discriminating non-coding regions of DNA called Short Tandem Repeats (STRs) (1). These STRs can be visualized from DNA profiles on an electropherogram produced by Capillary Electrophoresis (CE) (3). As such, a typical DNA typing workflow consists of DNA extraction, quantitation, amplification, and CE (4).

1.2 DNA Extraction

The success of generating an interpretable DNA profile is limited by the efficient extraction of DNA from cells (5). An efficient extraction procedure is one that successfully removes inhibitors that can affect downstream PCR analysis while simultaneously preventing the loss of DNA during purification (6).

1.2.1 DNA Extraction Chemistries

Mammalian cells are composed of a plasma membrane made up of a protein-lipid bilayer that forms a barrier between intracellular and extracellular components (7). Water is the most common molecule present in the membrane and is hydrogen-bonded to the polar headgroup region of the phospholipid bilayer (8). In cellular lysis, the cell membrane is destroyed and intracellular material, such as DNA, is released (7). Cell lysis methods can be achieved either by physical-, detergent-, or enzymatic-based disruption (7).

1.2.1.1 Physical Disruption

In physical disruption, the cell membrane is lysed by external forces to release cellular components. These forces include sonication, heat, and osmotic pressure. However, physical disruption using high heat has the potential to denature proteins (7).

1.2.1.2 Detergent-based Disruption

Detergent-based disruption is a milder alternative to physical disruption, where the protein-lipid bilayer of cells is broken through the solubilization of proteins and disruption of lipid-lipid, protein-protein, and protein-lipid interactions (7). Like the lipid bilayer that forms the plasma membrane of cells, detergents have non-polar tail groups that have hydrophobic-associating properties (9). In low concentrations, detergents bind to the membrane of cells by partitioning into the lipid bilayer (9). As a result, detergents allow the mixing of hydrophobic compounds into aqueous media, leading to the solubilization of membrane proteins. There are many common detergents used in protein extraction and the decision of which detergent to use is dependent on the biochemical properties of the target proteins and downstream applications (9). One common detergent that works on most cell types is Sodium Dodecyl Sulfate (SDS), whose anionic property adds an overall negative charge to all proteins in the sample, leading to cell lysis (9).

1.2.1.3 Enzymatic-based Disruption

Enzymatic-based disruptions utilize proteases such as Proteinase K and EA1 to hydrolyze peptide bonds and digest structural proteins and enzymes. Proteases are a class of enzymes that cleave peptide bonds in proteins. High temperatures that are often required for cellular lysis can also lead to enzyme deactivation. As such, thermophilic enzymes that can withstand high temperatures are favorable for assisting cellular digestion (10). Thus, for thermophilic enzymatic-based disruptions, there is typically a heating step to activate the enzyme, followed by another heating step at a higher temperature to inactivate the enzyme before PCR amplification so as not to affect downstream analysis.

Proteinase K, derived from *Tritirachium album* Limber, is a specific protease that acts by binding to proteins of interest through non-specific hydrophobic interactions (11,12). It then undergoes an activation step that leads to the formation of an active site that can cleave peptide bonds on the carboxylic acid side of amino acid residues (11). After cleavage, peptides are released from the enzyme. Proteinase K is optimal at a temperature of around 60 degrees Celsius (°C). Proteinase K is then heated to 95°C so that it becomes inactivated (11). The activity of Proteinase K is also enhanced in the presence of SDS (11).

Isolated from Mount Erebus, Antarctica, *Bacillus* sp. strain EA1 is a thermostable protease that has an optimal activation temperature of 75°C and a deactivation temperature of 95°C (10). EA1 protease works by preferentially cleaving the N-terminal side of hydrophobic or bulky amino acid side chains (13).

1.2.2 Inhibition

Red blood cells contain an iron-containing molecular component that is known as heme, which is a common PCR inhibitor (5). Inhibitors prevent successful PCR amplification through different mechanisms such as through binding to the polymerase, interaction with DNA, and interaction with the polymerase during primer extension (14). In quantitative PCR (qPCR), the threshold cycle (C_t) is the PCR cycle number at which the emitted fluorescence from the sample exceeds the threshold value – the greater the amount of starting DNA, the sooner the threshold cycle is reached (15). An internal positive control (IPC) is typically included in PCR kits to detect the presence of inhibitors in the sample (16). The IPC value is the same in every sample unless there is an inhibitor slowing down its amplification, which causes a larger IPC value (15). Thus, inhibitors must be removed prior to PCR to ensure an interpretable STR profile.

The silica-binding method is a purification method that uses a silica membrane that DNA binds to in the presence of chaotropic salts and a low potential of hydrogen (pH) (5). After a series of wash steps and tube transfers, the DNA is then eluted off the silica column by lowering the concentration of chaotropic salts and raising the pH of the solution, leading to purified and PCR-ready DNA in the solution (5). However, this method is preferred for pristine samples with little DNA degradation as there is a substantially lower DNA yield due to loss of DNA in the wash steps and failure of elution of DNA off the silica column when DNA is degraded (17).

As forensic laboratories typically encounter limited amounts of biological material with degraded DNA, direct lysis procedures are the preferred option for poor samples due to a lack of tube transfers and the absence of a purification step, where a high amount of DNA can be lost (18,19). Additionally, the workflow of direct lysis methods is typically quicker and easier, which is beneficial for laboratories facing the challenge of a high work volume (18,19). Direct lysis methods extract DNA using lysis buffers that disrupt cell membranes and release DNA into the lysate solution that is then used for PCR analysis (18). However, the lack of a purification step also means that impurities that may inhibit downstream analysis do not get removed prior to PCR analysis.

1.2.3 Arcturus® PicoPure® DNA Extraction

The Arcturus® PicoPure® (ThermoFisher, Waltham MA) DNA Extraction kit is a single-tube extraction that is ideal for processing small samples since DNA is not lost due to organic extractions or spin columns (20). An optimized Proteinase K extraction procedure using DNA Reconstitution Buffer is used to produce PCR-ready samples (20). The general protocol involves incubation of the sample with Proteinase K mixed with DNA Reconstitution Buffer for three hours at 65°C followed by a ten-minute incubation at 95°C to deactivate the Proteinase K before PCR analysis (20). PicoPure® is typically used on single cells that have been isolated using Laser Capture Microdissection (LCM) (21).

1.2.4 Chelex®-100 Resin Extraction

Chelex®-100 (Bio-Rad Laboratories, Hercules CA) is a chelating resin that is made of styrene-divinylbenzene copolymers that contain paired iminodiacetate ions which act as chelators for polyvalent metal ions (22). Chelex® utilizes heat and osmotic lysis to break open cells. The presence of Chelex resin during the boiling step that initiates cellular lysis prevents the degradation of DNA by chelating metal ions that assist in the degradation of DNA at high temperatures (22). Thus, extracellular components and inhibitors are bound to Chelex resin at the bottom of the tube while single-stranded DNA is left in the supernatant. The supernatant is then subsequently analyzed using PCR.

1.2.5 Casework Direct System Extraction

The Casework Direct System (Promega, Madison WI) is a quick and streamlined method of extraction without the need for purification of the lysate (23). The Casework Direct System kit contains Casework Direct Reagent of undisclosed formulation, along with 1-Thioglycerol as a reducing agent. Reducing agents are added to the buffer to avoid loss of protein or enzyme activity that is caused by oxidation (24). Thiol-containing compounds have been shown to be effective at protecting DNA from irradiative damage (25). The general Casework Direct System Extraction protocol consists of incubation of the sample with 1-Thioglycerol and Casework Direct Reagent for 30 minutes at 70°C in a spin basket. After 30 minutes, the contents of the basket are spun down, leaving the substrate in the basket and PCR-ready lysate in the tube (23).

1.2.6 Chai Enzymatic DNA/RNA Extraction

The Chai Enzymatic DNA/RNA Extraction kit (Chai, Santa Clara CA) includes a buffer with enzymes that are active at ambient temperatures and inactivated at 98°C (26). These enzymes break down nucleases that are present in the sample that may impede extraction, digest extracted DNA, or inhibit PCR analysis. As a result, no spin column purification or organic solvents are needed for sample processing prior to PCR analysis. The protocol includes a 15-minute incubation of the sample and DNA/RNA extraction buffer at room temperature followed by a 5-minute incubation at 98°C to inactivate the enzymes prior to PCR analysis (26).

1.3 DNA Recovery

Typically, 0.75 nanograms (ng) of DNA are required to generate an optimal DNA profile (27). Profiles generated from a low amount of DNA are subject to stochastic effects such as allelic dropout and drop-in, low relative fluorescence units (RFUs), and variable stutter peak heights (28). Thus, it is important that as many cells as possible are collected from forensic samples, eluted off the substrate, and lysed so that a usable DNA profile is produced (28).

1.3.1 Cellular Adherence and Release

When presented with evidence, an investigator or analyst must decide on how that evidence is collected (29). If biological fluid is found on a piece of clothing, a portion of the sample can be cut directly from the fabric and the swatch can then be used in DNA analysis. Conversely, if biological fluid is found on a hard surface such as a countertop or floor, an investigator may choose to swab the biological fluid off the surface. The swab will then be used for downstream analysis. The latter method introduces the problem of how well the cells are transferred from the original hard surface onto the swab. Both methods introduce the problem of determining how well DNA is recovered from cells that have been transferred onto the fabric or swab. It has been shown that approximately 20%-76% of DNA that is collected by a cotton swab is lost during extraction (30). Thus, there is a fine line between developing a method where a substantial number of cells adhere to a swab when being collected from a surface while also being able to remove all the cells from the substrate for DNA extraction.

1.3.3 Theoretical Yield Calculations

Extraction methods can be evaluated through a comparison of an actual yield to a pre-calculated theoretical yield (27). A theoretical yield is an estimation of the total amount of DNA that a sample had prior to extraction. Direct lysis methods are optimal for determining a theoretical yield as cells and/or DNA are not lost during multiple wash steps and/or tube transfers. In theoretical yield calculations, it is assumed that all the cells in the sample are lysed and present in the lysate.

*1.3.3.1 Acrosolv and forensic*GEM™

Acrosolv and *forensic*GEM™ can be used to extract samples for theoretical yield calculations. *Acrosolv* is a proprietary mixture of reagents in the *forensic*GEM™ Sperm kit that weakens cell walls at an optimal temperature of 52°C, releasing DNA into solution. In addition to *Acrosolv, forensic*GEM™, containing the thermophilic enzyme EA1 proteinase, is added to the solution to aid in the lysing of cells as well as removing nucleoproteins from DNA, making the sample suitable for downstream PCR analysis without the need for a purification step (31). Although *Acrosolv* is included in the *forensic*GEM™ Sperm kit, it can be used with blood to ensure full lysis of red blood cells.

1.4 Fabrics as Forensic Substrates

A fiber is a small, hair-like natural or manufactured substance that can be processed into fabric. Fibers can be either man-made from regenerated or synthetic material or can be natural from animal, plant, or mineral material (32). Yarn is an assemblage of continuous strands of fibers that are twisted or laid together to form a strand that can be made into a fabric. Therefore, fabric is a flexible planar structure that is produced by interlacing filaments, fibers, or yarn in any combination. Woven fabrics are made by interlacing two sets of yarn, knit fabrics are made by an interlocking series of loops of one or more yarns, and nonwoven fabrics are made by thermal, mechanical, solvent, or chemical means (32).

It is possible that the different compositions of fabrics may affect the ability of cells to adhere to and release from fabrics. Woven fabrics generally have yarns that are held together more loosely, while knit and nonwoven fabrics have yarn that are held together more tightly (32). Another factor that may influence the ability of cells to attach and detach from fabric could be thread count. Thread-count is the sum of all the threads in both the length and width directions contained in one square inch of fabric and is therefore a measurement of the coarseness or fineness of a fabric (32). Low thread-count fabrics have fewer groups of fibers per unit length than high thread-count fabrics.

1.4.1 Cotton

Cotton is the most widely produced natural fiber on the planet (33). Cotton swabs are also the most used type of swab in a forensic setting (34). Cotton fibers are comprised of cells from hairs that are on the outer layer of cotton plant seeds and are composed of 88.0%-96.5% α-cellulose (35). Cellulose fibers chemically consist of linked glucose units that contain free hydroxyl groups (36). This makes cotton more capable of strong molecular bonding with water and hydrophilic phosphate heads that are present on cellular membranes, leading to a greater affinity for bonding and potentially a lower affinity for the release of cells (32).

1.4.2 Rayon

Rayon is a man-made cellulose fiber that has similar properties to that of cotton and is commonly used for clothing, window drapes, and cooling sheets (32,36). Viscose rayon is treated so that the hydroxyl groups are replaced with acetyl groups, potentially causing reduced affinity to cell membranes (37,38). Viscose rayon is made from cellulose by dissolving wood chips and pressing dissolved wood pulp between rollers.

1.4.3 Nylon

It is believed that conventional cotton swabs used in crime laboratories can trap cellular materials within their fibers. As a result, nylon-flocked swabs have been introduced as an alternative collection device (34). Nylon, which is also known as polyamide, is a man-made synthetic polymer that is composed of amide bonds that are capable of strong hydrogen bonding with nucleic acid chains (36). Nylon has been used routinely as suture material and cell culture substrate due to its hydrophilicity with the water in cell membranes (39).

1.4.4 Wool

Wool is a natural animal fiber that comes from sheep. It is composed of α-keratin proteins, which are made up of α-amino acids. The external makeup of a wool fiber has a waxy cuticle layer, making wool water-repellent while allowing the absorption of water vapor (37). Once water travels to the cortex via the cell membrane complex, wool has high moisture-absorbing properties and a high affinity for nucleic acid chains due to the large number of amino groups on the hair's macromolecular chains (40).

1.5 Purpose of this Study

Experiments reported in this study investigated DNA yield from whole blood deposited on cotton, nylon, wool, rayon, or cotton swabs using various direct lysis extraction methods. The purpose of this study is to understand the relationship between DNA recovery from various fabrics with different compositions and direct lysis methods with differing cell membrane disruption protocols. The methods compared are PicoPure®, Chai, Casework Direct, and Chelex®.

2. MATERIALS AND METHODS

2.1 Theoretical Yield

The theoretical yield from liquid whole blood was studied. All equipment, pipettes, and benchtops were cleaned with 10% Sodium Hypochlorite (bleach) followed by 70% ethanol.

2.1.1 Blood Preparation

Single-donor human whole blood a tube containing Ethylenediaminetetraacetic acid (EDTA) was obtained. The blood was inverted gently, aspirated, and unaspirated several times to homogenize the sample.

2.1.2 Sample Preparation

Cotton, nylon, wool, and viscose rayon were obtained from TestFabrics Inc (TestFabrics, Pittston, PA). "International Organization for Standardization (ISO) adjacent" fabrics, meaning that each fabric contains properties that are in accordance with ISO 3801, were chosen to eliminate differences in warp and weft dimensions and the core structure of each fabric. The ISO 3801-accordant properties and their values for each fabric are listed in **Table 1**.

Table 1. Fabric Properties. Each fabric is in accordance with ISO 3801.

Fabric	Mass per unit area	Luminance (Y_{10})	Whiteness (W_{10})	Tint (T_{10})	pH
Cotton	(115 ± 5) g/m^2	89 ± 2	80 ± 3	-1 ± 1	7 ± 0.5
Nylon	(130 ± 5) g/m^2	86 ± 2	65 ± 2	1 ± 1	7 ± 0.5
Wool	(125 ± 5) g/m^2	72 ± 2	—	—	7.5 ± 0.5
Rayon	(140 ± 5) g/m^2	85 ± 5	75 ± 6	-1 ± 1.2	7 ± 0.5

The fabrics were placed onto a sheet of Nalgene™ VersiDry™ Surface Protector (Thermofisher, Waltham, MA) and cut into four 4 centimeter (cm) by 6 cm swatches. Two 5 µL drops of blood were deposited onto each swatch. An additional 5 µL of blood were deposited directly onto eight cotton swabs, The swatches and cotton swabs were left to dry overnight in a biohazard hood.

Cuttings of each complete dried stain along with a 5 millimeter (mm) by 5 mm cutting of each fabric containing no stain (fabric control) were taken and deposited into appropriately labeled sterile tubes. Dimensions of the stained cuttings were not noted due to the varying spread of blood on each fabric. The tubes were then stored at -30°C until extraction.

2.1.3 *Acrosolv* and *forensic*GEM™ Extractions

Five microliters (µL) of 17 samples of neat and 13 samples of 1:10 diluted whole blood were extracted at different time points along the course of this experiment for a total sample size of thirty.

The *forensic*GEM™ Universal kit was used (Hamilton, New Zealand), and the samples were extracted following the *forensic*GEM™ Handbook (41). Five µL per sample of whole blood, 10 µL per sample of 10X RED+ buffer, 1 µL per sample of *forensic*GEM™, and 84 µL per sample of Tris-EDTA (TE) buffer (10 millimolar (mM) Tris-Hydrochloride (HCl), 0.1mM EDTA, pH 8.0) were added into three 0.5 milliliter (mL) tubes. The samples were gently mixed by inversion of the tubes. The samples were transferred to the thermocycler, which was preheated to 75°C and underwent a 5-minute incubation at 75°C, followed by a 5-minute incubation at 95°C. The samples were then centrifuged for 5 minutes at 13,000 RCF to pellet extracellular material. The supernatant containing the DNA was then transferred into three new 0.5 mL tubes and used for PCR analysis.

The *forensic*GEM™ Universal kit was again used and three additional samples of neat whole blood and two additional samples of 1:10 diluted blood were extracted following the *forensic*GEM™ Handbook using the aforementioned procedure (41). After extraction, the samples were vortexed vigorously and the mixed extract was used for PCR analysis.

A combination of the *forensic*GEM™ Universal kit and the *forensic*GEM™ Sperm kit was used (Hamilton, New Zealand), and 22 samples were extracted in triplicate following the *forensic*GEM™ Sperm Handbook (31). Five µL per sample of neat or 1:10 diluted whole blood, 10 µL per sample of 10X RED+ buffer, 2 µL per sample of *forensic*GEM™, 10 µL of *Acrosolv,* and 73 µL per sample of TE buffer (10mM Tris-HCl, 0.1mM EDTA, pH 8.0) were added into 22 0.5 mL tubes. The samples were gently mixed by inversion of the tubes. The samples were transferred to the thermocycler, which was preheated to 52°C and underwent a 10-minute incubation at 52°C, followed by a 6-minute incubation at 75°C and finally a 3-minute incubation at 95 °C. The samples were then vortexed in preparation for PCR analysis.

2.1.4 Quantitation Using Quantifiler® Trio

The samples were quantified in duplicate using the Quantifiler® Trio DNA Quantification Kit (Applied Biosystems®, Foster City, CA) and the QuantStudio™ 5 Real-time PCR System for Human Identification (Applied Biosystems®, Foster City, CA). Quantification was carried out using the ABI Quantifiler® HP and Trio DNA Quantification Kits User Guide (42). A Master Mix of Quantifiler Trio Primer Mix (8 µL/reaction) and Quantifiler Trio PCR Reaction Mix (10 µL/reaction) was prepared. Eighteen µL per sample of Master Mix were added to wells on a 96-well plate. Two µL per sample of each sample were added to the 96-well plate to wells containing the Master Mix. The QuantStudio™ 5 Real-Time PCR Instrument was run according to the QuantStudio™ 5 Real-Time PCR Instrument (for Human Identification) User Guide (43). Each experiment was run with one negative blank control and two positive reagent controls.

2.2 Direct Lysis Recovery Study

2.2.1 Arcturus® PicoPure® DNA Extraction

Arcturus® PicoPure® DNA Extractions were performed in duplicate. Each stained fabric swatch and cotton swab were placed into appropriately labeled 0.5 mL tubes. For each sample, one vial of Proteinase K was centrifuged for 30 seconds at 1000 relative centrifugal force (rcf). One hundred fifty-five µL of Reconstitution Buffer was added to the vial of Proteinase K. The tube was then inverted gently to mix the reagents completely. One hundred fifty µL of the mixture was then added to each tube containing a fabric swatch or cutting. The tubes were inverted gently to mix the cells thoroughly and then incubated at 65°C for three hours. The sample tubes were then incubated at 95°C for ten minutes to inactivate the Proteinase K.

The extracts were quantified in triplicate using the method described in 2.1.3.

Swabs and cuttings extracted with PicoPure® were placed in Investigator Lyse&Spin Baskets, (Qiagen, Hilden, Germany), and dried through centrifugation for the DNA loss study.

2.2.2 Chelex® 100 Resin Extraction

Chelex® 100 Resin Extractions were performed in duplicate. Each fabric swatch and cotton swab with dried blood was placed into appropriately labeled 0.5 mL tubes. One hundred µL of TE buffer were added into each tube and the samples were incubated at room temperature for 23 minutes. The samples were occasionally vortexed gently during this incubation.

A 5% Chelex® 100 Suspension was prepared by adding 50 mL of deionized water and 2.5g of Chelex to a capped tube. The 5% Chelex was vortexed thoroughly to ensure proper mixing of the resin beads in the solution. One hundred μL of 5% Chelex were added to the tubes to bring the final volume to 200 μL. The samples were then incubated at 56°C for 23 minutes. After incubation, the sample tubes were vortexed at high speed for ten seconds. The sample tubes were then incubated at 100°C for 8 minutes and subsequently vortexed. The sample tubes were spun for 3 minutes at maximum angular velocity.

The supernatants of the extracts were quantified in triplicate using the method described in 2.1.3.

Swabs and cuttings extracted with Chelex® were placed in Investigator Lyse&Spin Baskets and dried through centrifugation for the DNA loss study.

2.2.3 Casework Direct System Extraction

In a preliminary study using Casework Direct, 5 μL of neat whole blood were deposited onto 2 cotton swabs. One-Thioglycerol was diluted tenfold in water by adding 30 μL 1-Thioglycerol to 270 μL water. One μL of diluted 1-Thioglcycerol was added to 200 μL of Casework Direct Reagent to make Casework Direct solution. Two hundred μL of Casework Direct Solution were added to each basket containing a fabric swatch or swab. Each tube containing the spin basket was vortexed for 10 seconds and then placed on a heat block to incubate at 70°C for 30 minutes. The tubes containing the spin baskets were vortexed for 10 seconds and then centrifuged at room temperature for 5 minutes at maximum speed. The extracts were amplified using qPCR and yielded data with IPC values marked as "Undetermined".

An additional preliminary study was done on 5 µL of neat whole blood deposited onto 4 cotton swabs. The swabs were extracted using the aforementioned procedure. Prior to qPCR, 2 Casework Direct extracts were diluted tenfold while the other 2 were left undiluted. The undiluted Casework Direct extracts yielded data with IPC values marked as "Undetermined"

For the main experiment, Casework Direct System extractions were performed in duplicate. Each stained fabric swatch and cotton swab were placed into appropriately labeled Investigator Lyse&Spin Baskets. All fabrics and swabs were extracted using the procedure described above.

Based on the data from the preliminary study, all Casework Direct extracts were diluted tenfold and then quantified in triplicate using the method described in 2.1.3. All future Casework Direct data from this study was obtained from the Casework Direct extracts that were diluted tenfold prior to qPCR.

The spin baskets containing the cuttings extracted with Casework Direct were removed and placed in sterile tubes for the DNA loss study.

2.2.4 Chai Enzymatic DNA/RNA Extraction

Chai Enzymatic DNA/RNA Extractions were performed in duplicate. Each stained fabric swatch and cotton swab were placed into appropriately labeled 0.5 mL tubes.

Two hundred μL of Enzymatic DNA/RNA Extraction Buffer 1X were added to the tube containing a fabric swatch or swab. The sample and buffer were mixed completely by placing the tube on a vortex for 15 seconds. The sample was then incubated at ambient temperature for 15 minutes then 98°C for 5 minutes.

The swabs and fabric cuttings were placed into Investigator Lyse&Spin Baskets, centrifuged at maximum r.c.f. for 5 minutes, and placed in sterile tubes for the DNA loss study.

The extracts were quantified in triplicate using the method described in 2.1.3.

Swabs and cuttings extracted with the Chai Enzymatic DNA/RNA Extraction kit were placed in Investigator Lyse&Spin Baskets and dried through centrifugation for the DNA loss study.

2.2.5 Percent Yield Calculation

The DNA concentrations (ng/ μL) that were given by the QuantStudio™ 5 Real-time PCR Instrument for all sample replicates of each condition were averaged. The various conditions with the amounts of replicates are displayed in **Table 2**. Only the blood samples diluted to 1:10 and extracted with *Acrosolv* were averaged and used as the standard value because diluted blood, when dilution is factored out, yielded a higher mass of DNA than neat blood. These results are explained in 3.2. The amount of DNA in ng for each condition was calculated by multiplying the average qPCR concentration of each condition by the final elution volume of the lysate:

qPCR concentration (ng/μL) × Elution Volume (μL) = Amount of DNA (ng)

The percent yield was calculated by dividing the average amount of DNA in ng of the various sample conditions by the average amount of DNA in ng extracted from the diluted whole blood samples using *Acrosolv*. This value was then multiplied by 100%.

$$\frac{\text{DNA Amount (ng) from each Condition}}{\text{1:10 Blood Dilution Extracted with } Acrosolv \text{ DNA Amount (ng)}} \times 100\% = \text{Percent Yield}$$

Table 2. Experimental Conditions. Each condition was run in duplicate (1-2) along with one negative control (NTC). qPCR values of each duplicate were averaged. All Casework Direct extracts were diluted 1:10 prior to qPCR.

	PicoPure (PP)	Chai (A)	Chelex (X)	Casework Direct (D)
Cotton (C)	C-PP-1 C-PP-2 C-PP-NTC	C-A-1 C-A-2 C-A-NTC	C-X-1 C-X-2 C-X-NTC	C-D-1 C-D-2 C-D-NTC
Nylon (N)	N-PP-1 N-PP-2 N-PP-NTC	N-A-1 N-A-2 N-A-NTC	N-X-1 N-X-2 N-X-NTC	N-D-1 N-D-2 N-D-NTC
Wool (W)	W-PP-1 W-PP-2 W-PP-NTC	W-A-1 W-A-2 W-A-NTC	W-X-1 W-X-2 W-X-NTC	W-D-1 W-D-2 W-D-NTC
Rayon (R)	R-PP-1 R-PP-2 R-PP-NTC	R-A-1 R-A-2 R-A-NTC	R-X-1 R-X-2 R-X-NTC	R-D-1 R-D-2 R-D-NTC
Cotton swab (S)	S-PP-1 S-PP-2 S-PP-NTC	S-A-1 S-A-2 S-A-NTC	S-X-1 S-X-2 S-X-NTC	S-D-1 S-D-2 S-D-NTC

2.3 DNA Loss Study

To assess each direct lysis method's ability to extract DNA from the samples, re-extractions of each previously extracted substrate were performed.

All equipment, pipettes, and benchtops were cleaned with 10% Sodium Hypochlorite (bleach) followed by 70% ethanol.

2.3.1 Re-Extraction of Substrates

All samples except for those extracted with Chelex were re-extracted using a variation of the *Acrosolv* and *forensic*GEM™ extraction procedure outlined in 2.1.2. Spin baskets containing each dried, previously extracted substrate were added into 30 sterile 2 mL tubes. For each sample, 20 µL of 10X RED+ Buffer, 4 µL of *forensic*GEM™, 20 µL of *Acrosolv,* and 156 µL of autoclaved TE buffer (10mM Tris-HCl, 0.1mM EDTA, pH 8.0) were placed into the spin basket containing the substrate. The samples were gently mixed and placed on a Thermo Scientific™ Thermal Mixer (Fisher Scientific, Waltham, MA) at 400 r.p.m. and incubated for 10 minutes at 52°C, 6 minutes at 75°C, and 3 minutes at 95 °C.

The re-extracted lysates were each quantified once using the method described in 2.1.3.

2.3.2 Percent Recovery Calculation

The amount of DNA in ng left behind on the substrate after the initial extraction was calculated by dividing the mass of DNA recovered after initial extraction by the sum of the amount of DNA recovered after the initial extraction and re-extraction and multiplying the value by 100%.

$$\frac{\text{Initial extraction DNA Amount (ng)}}{\text{Initial extraction+re−extracted DNA Amount (ng)}} \times 100\% = \text{Percent Recovery}$$

2.4 Statistical Analysis

Two-way analysis of variance (ANOVA) was performed to evaluate the relationship between the means of the initial mass of DNA in ng obtained from the fabrics and direct lysis methods.

Several One-way ANOVAs were performed to compare the following: the difference in means of the masses of DNA in ng after initial extraction between each fabric, the difference in means of the masses of DNA in ng after re-extraction between each fabric, the difference in means of the masses of DNA in ng after initial extraction between each direct lysis method, the difference in means of the masses of DNA in ng after re-extraction between each direct lysis method.

One-way ANOVA was also performed to compare IPC and small autosomal values between neat and 1:10 dilutions of whole blood extracted during the theoretical yield study as well as compare IPC and small autosomal values from the fabric and direct lysis study.

3. RESULTS

3.1 Comparison of DNA Mass from Varying Direct Lysis and Fabric Conditions

The mass of DNA in ng was calculated from the qPCR small autosomal (ng/µL) values obtained from PicoPure®, Chai, Casework Direct, or Chelex® extractions performed on 5 µL of blood deposited onto cotton, nylon, wool, rayon, and cotton swabs. All conditions were performed in duplicate and underwent qPCR analysis in triplicate. After initial extraction, all samples except for those extracted with Chelex, were re-extracted using *Acrosolv*. Samples extracted with Chelex® were not re-extracted due to low initial extraction amounts.

The mean small autosomal values (ng/ µL) and mass of DNA (ng) obtained from initial and re-extraction, as well as the total mass of DNA (ng) from each condition are displayed in **Table 3**.

Table 3. Small target quantitation data for PicoPure®, Chai, Casework Direct, and Chelex® extractions performed on Cotton, Nylon, Wool, Rayon, and Cotton Swabs. qPCR was performed on the original lysates from the described extraction methods and fabrics in triplicate. All Substrates except those extracted with Chelex were re-extracted using *Acrosolv*. Percent Remaining was calculated by dividing the Re-extracted Mass by the Total Mass.

		Initial Extraction		Re-extraction			
Direct Lysis Method	Fabric	Small Target Mean (ng/ µL)	Mass (ng)	Small Target Mean (ng/ µL)	Mass (ng)	Total Mass (ng)	Percent Remaining (%)
PicoPure®	Cotton	1.904	285.562	0.298	59.680	345.242	17.286
	Nylon	1.485	222.718	0.438	87.626	310.343	28.235
	Wool	1.690	253.431	0.322	64.474	317.905	20.281
	Rayon	1.663	249.391	0.144	28.760	278.151	10.340
	Cotton Swab	1.297	194.508	0.087	17.393	211.902	8.208
Chai	Cotton	0.885	176.991	0.117	23.394	200.385	11.675
	Nylon	0.601	120.129	0.071	14.263	134.392	10.613
	Wool	1.216	243.298	0.276	55.136	298.434	18.475
	Rayon	0.822	164.495	0.302	60.426	224.921	26.865
	Cotton Swab	0.759	151.856	0.246	49.225	201.081	24.480
*Casework Direct	Cotton	1.714	342.770	0.020	4.031	346.800	1.162
	Nylon	1.343	268.532	0.090	17.783	286.515	6.276
	Wool	1.722	344.387	0.019	3.775	348.162	1.084
	Rayon	1.732	346.421	0.027	5.397	351.818	1.534
	Cotton Swab	1.452	290.402	0.073	14.648	305.050	4.802
†Chelex®	Cotton	0.431	86.155	-	-	-	-
	Nylon	0.279	55.788	-	-	-	-
	Wool	0.562	112.437	-	-	-	-
	Rayon	0.541	108.204	-	-	-	-
	Cotton Swab	0.391	78.152	-	-	-	-

*Casework Direct extracts were diluted 1:10 prior to qPCR and dilution was factored out.
†Chelex® was excluded from this study due to low initial extraction amounts.

3.1.1 Two-way Analysis of Initial Total (ng)

A Two-way Analysis of the initial amount of DNA in ng obtained from cotton, nylon, wool, rayon, or cotton swabs extracted with PicoPure, Chai, Casework Direct, or Chelex was performed. A statistically significant difference in average DNA in ng obtained from both fabric (F Ratio = 3.2445, $p < 0.0332$) and direct lysis method (F Ratio = 41.9940, $p < 0.0001$) was found; however, the interaction between these conditions was not significant (F Ratio = 0.3993, $p < 0.9472$).

3.1.2 One-way Analysis of Initial Total (ng) by Direct Lysis Method

A One-way Analysis of the initial amount of DNA in ng obtained after performing each extraction method was performed. The difference in mass of DNA in ng initially extracted was statistically significant according to the direct lysis procedures (F-ratio = 40.0266, $p < 0.0001$). The means and standard deviations for each extraction method are shown in **Table 4**. Casework Direct had the highest mean of 318.502 ng of DNA while Chelex® had the lowest mean of 88.147 ng of DNA.

Table 4. Statistical Analysis assessing variance of initial total (ng) between direct lysis methods. Analysis of variance and standard deviation of the DNA mass obtained from PicoPure®, Chai, Casework Direct diluted tenfold, and Chelex®.

All Fabrics					
Direct Lysis Method	Sample Number	Mean DNA Mass (ng)	Standard Deviation	F Ratio	*p*-value
PicoPure®	10	241.122	55.319	40.0266	<0.0001
Chai	10	171.354	50.361		
Casework Direct	10	318.502	40.454		
Chelex®	10	88.147	49.179		

3.1.3 One-way Analysis of Re-extracted Total (ng) by Direct Lysis Method

A One-way Analysis of DNA re-extracted from substrates that had been initially extracted using each direct lysis method was performed. The difference in mass of DNA in ng recovered after re-extraction was statistically significant according to the direct lysis procedures (F-ratio = 4.1537 p = 0.0267). The means and standard deviations for each extraction method are shown in **Table 5**. Casework Direct had the lowest mean of 9.167 ng of DNA while PicoPure® had the highest mean of 51.587.

Table 5. Statistical Analysis assessing variance of re-extracted total between direct lysis methods. Analysis of variance and standard deviation of the DNA mass obtained from re-extraction of substrates that had been lysed using PicoPure®, Chai, or a 1:10 dilution of Casework Direct.

All Fabrics					
Direct Lysis Method	Number	Mean DNA Mass (ng)	Standard Deviation	F Ratio	*p*-value
PicoPure®	10	51.587	53.864	4.1537	0.0267
Chai	10	40.489	22.735		
Casework Direct	10	9.167	8.782		
*Chelex®	-	-	-		

* Chelex® was excluded from this study due to low initial extraction amounts.

3.1.4 Percentage of DNA Recovered by Each Direct Lysis Method

The mass and percentage of DNA obtained from the initial extraction and re-extraction from each direct lysis method are displayed in **Figures 1 and 2,** respectively**.** Casework Direct recovered the most initial and total mass of DNA, while Chai recovered the least initial and total mass of DNA. PicoPure® had the highest mass of DNA left behind on the substrate with the highest re-extraction quantity, while Casework Direct left the lowest mass of DNA on the substrate.

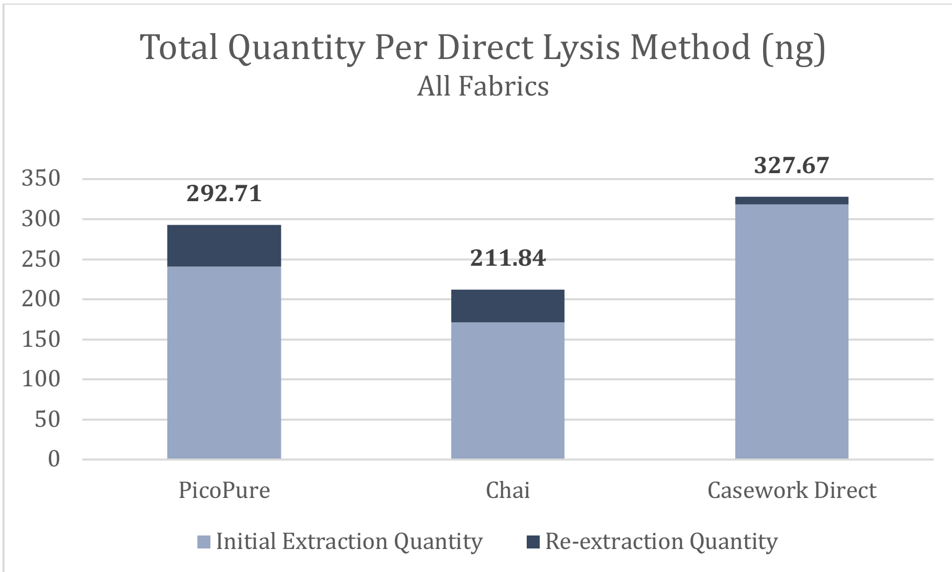

Figure 1. Initial and re-extracted mass of DNA in ng recovered using each Direct Lysis Method. Casework Direct extracts were diluted 1:10 prior to qPCR.

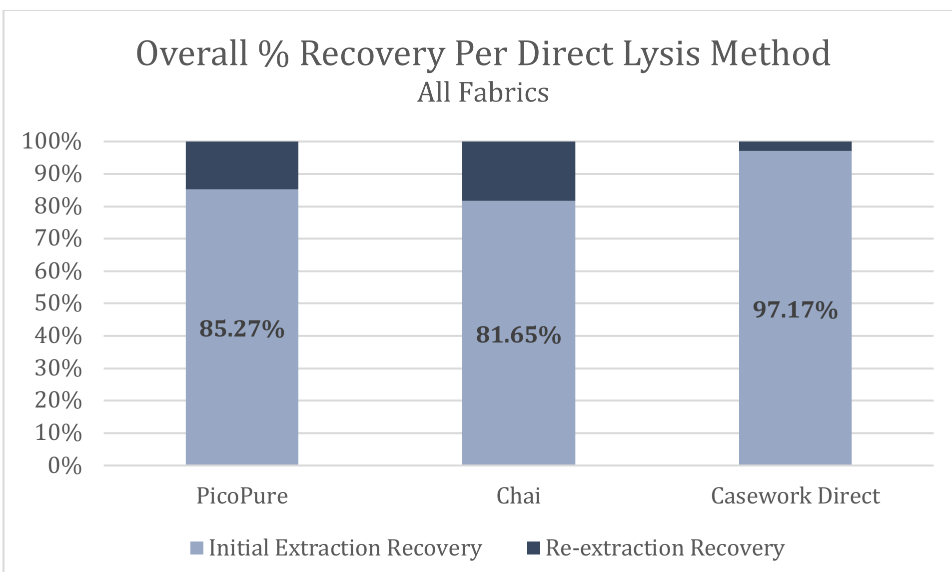

Figure 2. Percentage of DNA recovered using each direct lysis method. Casework Direct extracts were diluted 1:10 prior to qPCR.

3.1.5 One-way Analysis of Initial Total (ng) by Fabric

A One-way Analysis of the initial amount of DNA recovered from cotton, nylon, wool, rayon, and cotton swabs was performed. The mass of DNA in ng initially extracted was not significantly different according to fabric type (F ratio = 0.7532, p = 0.5627). The means and standard deviations for each extraction method are shown in **Table 6**.

Table 6. Statistical Analysis assessing variance of initial total between fabrics. Analysis of variance and standard deviation of the DNA mass obtained from cotton, nylon, wool, rayon, or cotton swabs.

All Direct Lysis Methods					
Fabric	Number	Mean DNA Mass (ng)	Standard Deviation	F Ratio	*p*-value
Cotton	8	222.869	116.163	0.7532	0.5627
Nylon	8	166.792	92.359		
Wool	8	238.388	99.129		
Rayon	8	217.128	101.007		
Cotton Swab	8	178.729	86.509		

3.1.6 One-way Analysis of Re-extracted Total (ng) by Fabric

A One-way Analysis of the amount of DNA recovered from re-extraction of cotton, nylon, wool, rayon, and cotton swabs was performed. The mass of DNA in ng initially extracted was not significantly different according to fabric type (F ratio = 0.1537, p = 0.9596). The means and standard deviations for each extraction method are shown in **Table 7**.

Table 7. Statistical Analysis assessing variance of re-extracted total between fabrics. Analysis of variance and standard deviation of the DNA mass obtained from re-extracted cotton, nylon, wool, rayon, or cotton swabs.

Acrosolv					
Fabric	Number*	Mean DNA Mass (ng)	Standard Deviation	F Ratio	*p*-value
Cotton	6	29.035	34.191	0.1537	0.9596
Nylon	6	39.957	60.879		
Wool	6	41.128	43.754		
Rayon	6	31.528	29.422		
Cotton Swab	6	27.089	19.355		

*Samples extracted with Chelex® not included.

3.1.7 Percentage of DNA Recovered from Each Fabric

The mass of DNA obtained from the initial extraction and re-extraction from each fabric is displayed in **Figure 3**. The highest total amount of DNA in ng was recovered from wool while the lowest amount was recovered from Nylon. However, the difference in DNA recovered from each fabric is negligible. The percentage of DNA recovered from each fabric can be found in **Figure A** in the appendix.

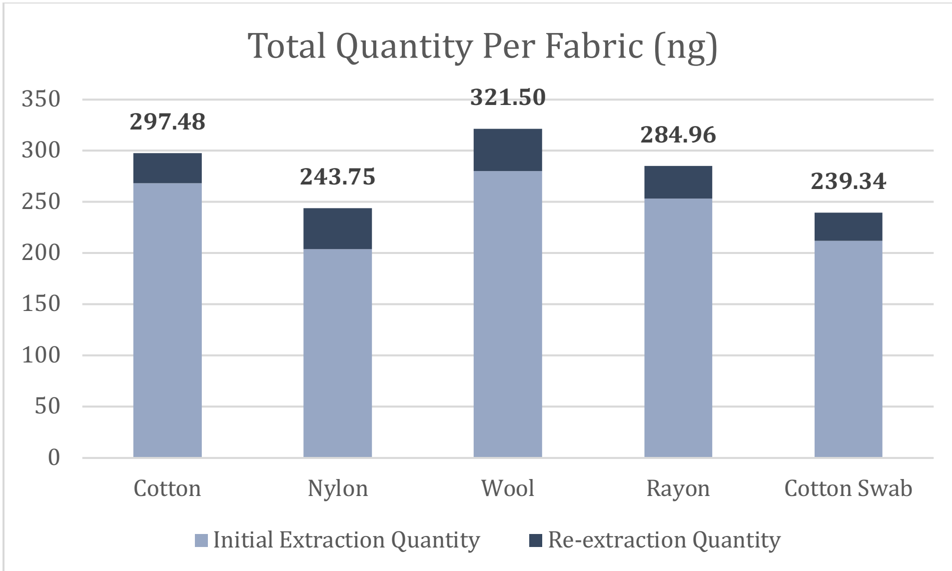

Figure 3. Initial and re-extracted mass of DNA in ng recovered from each fabric.

3.2 Quantitation of Theoretical Yield

As stated in 2.2.5, a total of thirty samples of 5 µL of neat and 1:10 blood dilutions were extracted using *Acrosolv* and *forensic*GEM™ with the goal of determining a theoretical amount of DNA in ng that is in 5 µL of whole blood. **Table 8** shows a summary of the amount of DNA recovered from neat and 1:10 blood dilutions and the final theoretical yield total in ng that was used for the percent yield calculations. However, because blood diluted tenfold and extracted with *Acrosolv* yielded the highest mass, the masses of only those samples were averaged to obtain a theoretical yield of 246.356 ng. Additionally, although *Acrosolv* and *forensic*GEM™ are direct lysis methods, it is possible that the final theoretical yield does not accurately reflect the initial amount of DNA in ng in 5 µL of neat whole blood.

Table 8. Small target quantitation data for *Acrosolv* and *forensic*GEM™ Extractions. qPCR was performed on Neat and 1:10 blood dilutions after running replicate samples. The mass of DNA in blood diluted tenfold and extracted using *Acrosolv* was averaged to determine the theoretical yield.

Acrosolv				*forensic*GEM™		
Dilution	Small Target (ng/ µL)	Mean (ng/ µL)	Mass (ng)	Small Target (ng/ µL)	Mean (ng/ µL)	Mass (ng)
Neat	1.828	1.778	177.803	1.245	1.575	157.459
	1.828			1.655		
	1.911			1.820		
	1.993			1.898		
	1.644			1.381		
	1.812			1.448		
	1.852			-		
	1.689			-		
	1.879			-		
	1.588			-		
	1.532			-		
1:10	1.408	2.464	246.356	1.414	1.286	128.563
	1.765			1.158		
	2.264			-		
	3.261			-		
	2.854			-		
	2.913			-		
	2.526			-		
	2.546			-		
	3.293			-		
	2.145			-		
	2.124			-		

3.2.1 Theoretical Yield Comparisons

After initial and re-extraction, the total mass of DNA in ng recovered from each direct lysis method performed on each swatch was averaged and is shown in **Table 9**. The initial mass for each direct lysis method was divided by the theoretical yield of 246.356 ng.

Casework Direct had the highest percent yield of 129.285% while Chelex had the lowest percent yield of 35.780%. **Figure 4** shows the initial mass of DNA obtained from each extraction method by fabric type.

Table 9. Comparison of actual yield to the theoretical yield. The initial mass (ng) of each direct lysis method was averaged and divided by the theoretical yield to obtain the percent yield of each direct lysis method. All Casework Direct extracts were diluted 1:10 prior to qPCR and dilution was factored out.

Direct Lysis Method	Fabric	Initial Mass (ng)	Mean Initial Mass (ng)	Mean Percent Yield (%)
PicoPure®	Cotton	345.242	241.122	97.875
	Nylon	310.343		
	Wool	317.905		
	Rayon	278.151		
	Cotton Swab	211.902		
Chai	Cotton	200.385	171.354	69.555
	Nylon	134.392		
	Wool	298.434		
	Rayon	224.921		
	Cotton Swab	201.081		
Casework Direct	Cotton	346.800	318.502	129.285
	Nylon	286.515		
	Wool	348.162		
	Rayon	351.818		
	Cotton Swab	305.050		
Chelex®	Cotton	86.155	88.147	35.780
	Nylon	55.789		
	Wool	112.437		
	Rayon	108.204		
	Cotton Swab	78.152		

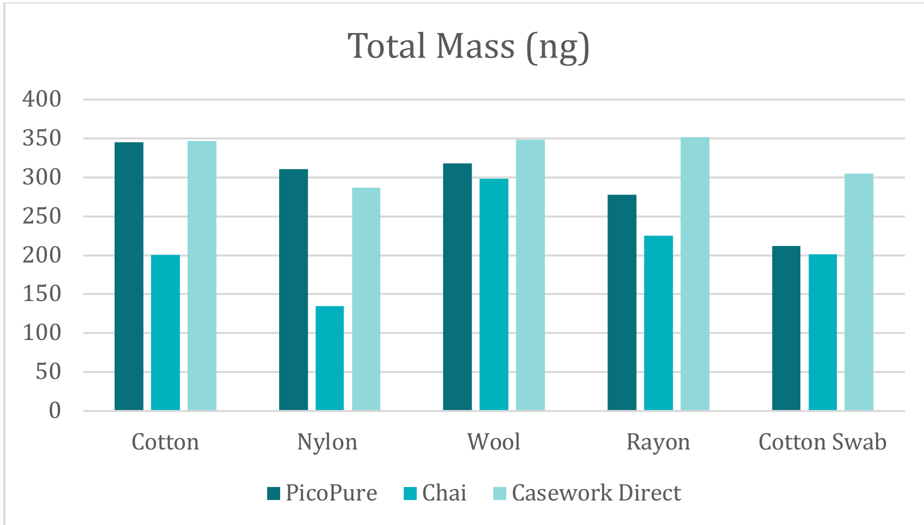

Figure 4. The total mass (ng) extracted using each direct lysis method by fabric type. Casework Direct extracts were diluted 1:10 prior to qPCR.

3.3 Inhibition Study

The IPC and small autosomal values of neat and 1:10 blood dilutions extracted with *Acrosolv* and *forensic*GEM™ as well as the IPC and small autosomal values of blood diluted with the direct lysis methods were compared to assess the possibility of inhibition of qPCR.

3.3.1 Neat and 1:10 Whole Blood Dilution Internal Positive Control Values

The IPC values for neat blood extracted using Acrosolv and *forensic*GEM™ were significantly higher than the IPC values for blood diluted to a 1:10 dilution (F Ratio = 49.4850, $p < 0.001$). The mean IPC value for neat blood was 28.272 and the mean IPC value for a 1:10 dilution of blood was 27.786. A box plot comparing the IPC values of neat and 1:10 dilutions of blood is displayed in **Figure 5**.

Small autosomal values calculated from neat blood were significantly lower than small autosomal values calculated from a 1:10 dilution of blood (F ratio = 10.320, p = 0.0033). The mean small autosomal value for the DNA concentration in neat blood samples was 1.706 ng/ µL while the mean small autosomal value for the DNA concentration in 1:10 dilutions of blood samples was 2.282 ng/ µL. A box plot comparing the small autosomal values of neat and 1:10 dilutions of blood is displayed in **Figure 6**.

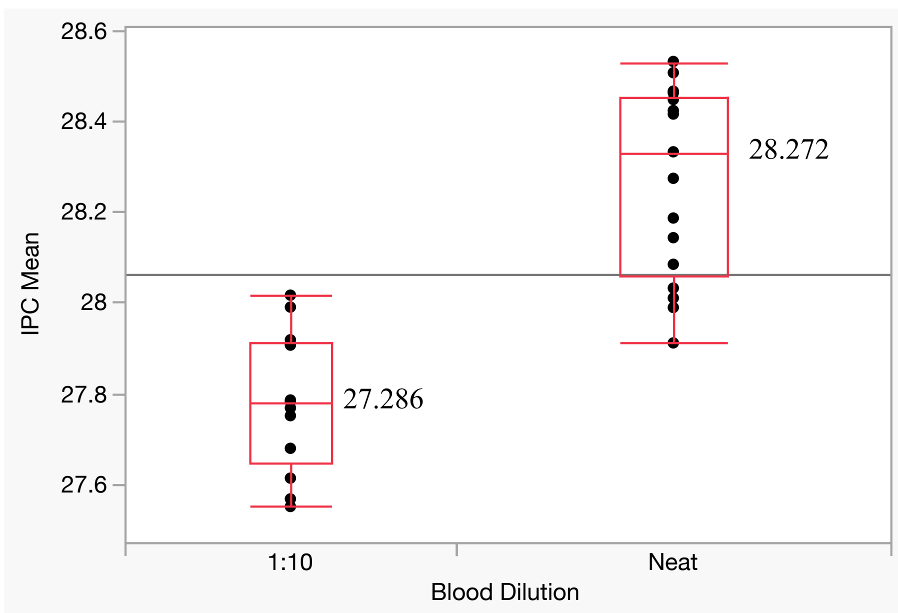

Figure 5. Comparison of IPC means by blood dilution – no fabric.

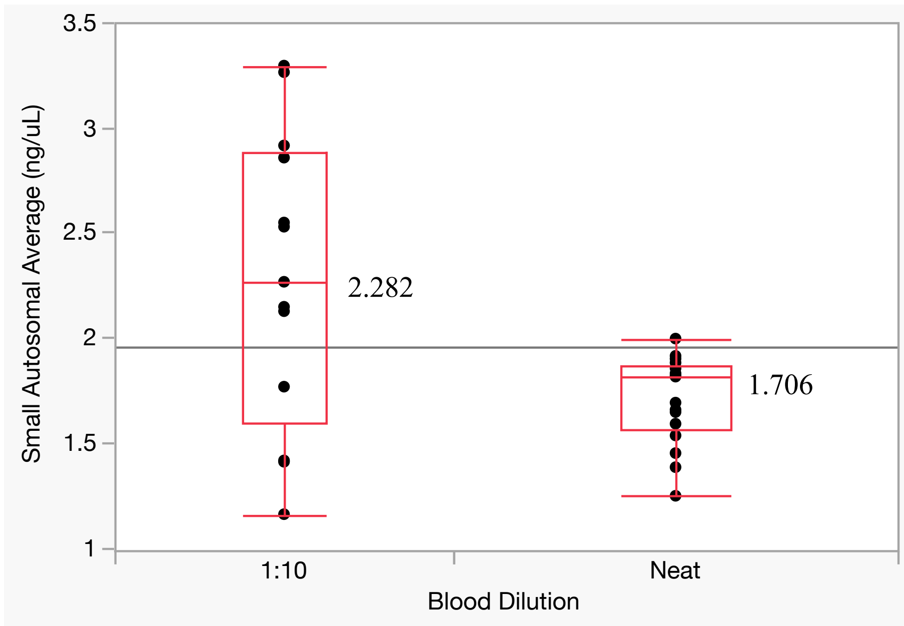

Figure 6. Comparison of small autosomal average values (ng/ µL) by blood dilution – no fabric.

3.3.2 Internal Positive Control Values for Direct Lysis Methods

The difference in IPC values between direct lysis methods was statistically significant (F-ratio = 4.1071, p = 0.0132). Casework Direct had the lowest IPC mean and the highest small autosomal (ng/µL) mean while Chelex had the largest IPC mean and the smallest small autosomal (ng/µL) mean. Box plots comparing IPC values and small autosomal values between direct lysis methods can be seen in **Figures 7 and 8**, respectively.

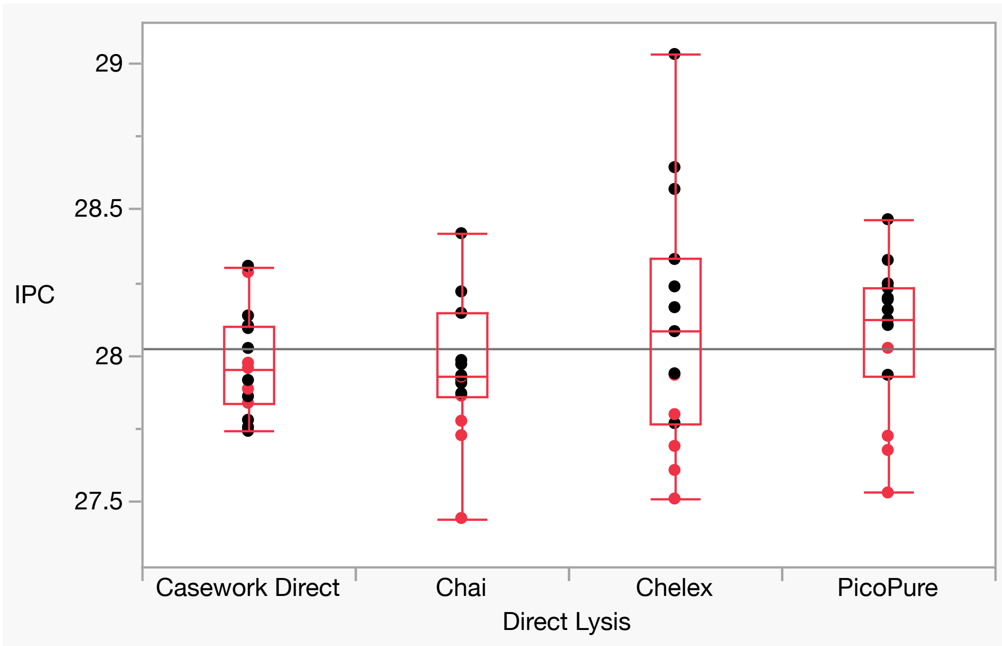

Figure 7. Comparison of IPC values by direct lysis method. NTC samples are marked in red. Casework Direct extracts were diluted 1:10 prior to qPCR.

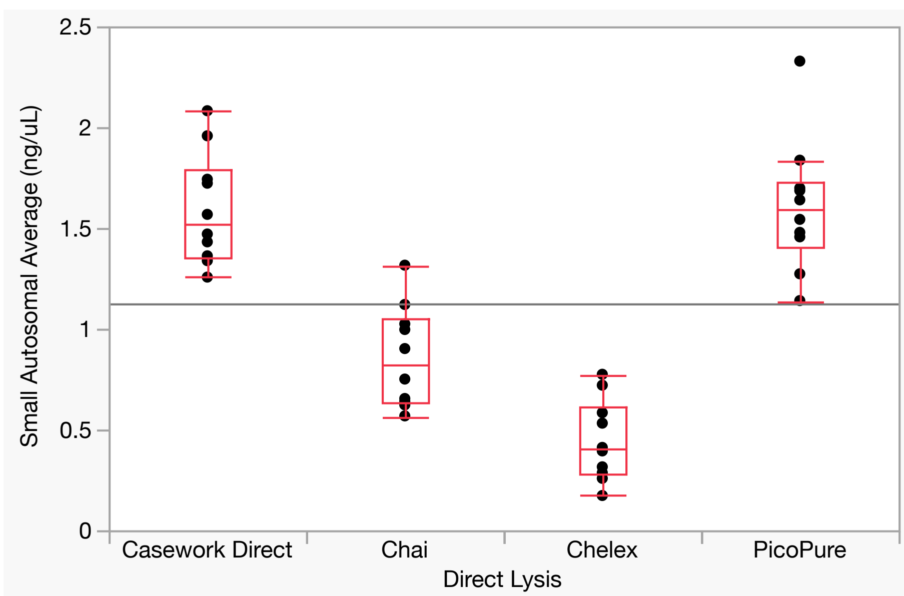

Figure 8. Comparison of small autosomal average values ng/µL by direct lysis method. Casework Direct extracts were diluted 1:10 prior to qPCR.

4. DISCUSSION

4.1 DNA Yield for Each Condition

4.1.1 Interaction Between the Direct Lysis Methods and Substrates

The lack of interaction between PicoPure®, Chai, Chelex®, or Casework Direct and cotton, nylon, wool, rayon, or cotton swabs suggests that fabric type does not affect the ability of the direct lysis methods to extract DNA from 5 μL of whole blood.

4.1.2 Direct Lysis Methods and the Initial Amount of DNA Extracted

Casework Direct recovered the highest amount of DNA after initial extraction compared to PicoPure®, Chai, and Chelex, while Chelex® recovered the lowest amount of DNA.

As stated previously, a Chelex® extraction requires a boiling step while Chelex® resin beads in the sample tube chelate metal ions that assist in DNA degradation at high temperatures. While no further experiments were conducted in this study, it is possible that an insufficient amount of resin beads was introduced to the sample tubes, causing an oversaturation of beads by metal ions, allowing degradation of DNA during the boiling step and thus, a low DNA yield. Chelex® was excluded from subsequent studies following this result.

4.1.3 Direct Lysis Methods and the Amount of DNA Recovered from Re-extraction

Substrates that had previously been extracted with Casework Direct yielded the lowest amount of DNA after re-extraction with *Acrosolv*. Thus, Casework Direct is the most efficient method for recovering DNA after an initial extraction in the methods studied. The Casework Direct protocol includes placing the substrate in a spin basket and drying it through centrifugation prior to PCR analysis while the other direct lysis methods do not call for this step. As a result, less DNA was left behind on the substrate after initial extraction.

Substrates that had previously been extracted with PicoPure® yielded the highest amount of DNA after re-extraction with *Acrosolv*. Thus, PicoPure® is the least efficient method for recovering DNA after an initial extraction. Because PicoPure® reagents are enclosed in vials with preset amounts, the highest volume a final elution volume can be is 155 µL. Based on observations of the samples during processing, substrates where blood spread out further were not completely saturated with such a small elution volume, causing a higher DNA yield when the same substrates were re-extracted using 200 µL of *Acrosolv*.

4.1.4 Fabrics and the Initial and Re-extracted Amounts of DNA Extracted

Fabric type had an insignificant effect on the amount of DNA recovered after initial extraction and the amount recovered after re-extraction with *Acrosolv*. Cotton, rayon, wool, and nylon share similar qualities (37). Cotton and rayon are both made from cellulose with free hydroxyl groups that have a high affinity for cellular membranes. Additionally, Nylon and wool both contain amino groups that also demonstrate strong hydrogen bonding with cells (37,44).

Although the difference is negligible, nylon had the lowest amount of DNA recovered from the fabric. Blood spread out and left the largest stain in comparison to other fabrics upon application of 5 µL of neat blood to nylon. As a result, the swatches of nylon placed into each tube were the largest compared to the other fabrics. It is possible that the nylon swatches were not submerged completely, leading to a lower DNA yield.

Wool had a slightly higher recovery compared to the other fabrics. Upon application of 5 µL of neat blood to wool, the blood did not spread out and appeared to stay on the surface. Because of this observation, it is possible that the blood dried before it could penetrate the waxy cortex of the wool. Since the blood did not penetrate as deeply into the wool as it did into the other fabrics, a higher amount of DNA was able to be extracted from the wool than from the other fabrics.

4.1.5 Percent Yield

Casework Direct had the highest percent yield and Chelex had the lowest percent yield when compared to the other direct lysis methods.

A theoretical yield is an estimation of the total amount of DNA in a sample assuming complete lysis of all cells. qPCR values using 5 μL of blood diluted tenfold and extracted with *Acrosolv* were used in generating a theoretical yield. Although *Acrosolv* and *forensicGEM*™ are direct lysis methods, there are possible areas of DNA loss in these protocols. For example, the *forensic*GEM™ protocol for liquid blood calls for the separation of the supernatant from the cell pellet, leading to possible DNA loss. Additionally, it may be that the proprietary reagents in *Acrosolv* and *forensic*GEM™ are not sufficient to successfully lyse and purify blood, while Casework Direct contains reagents that are more potent than that of *Acrosolv*, leading to a Percent Yield greater than 100%.

4.2 Impact of Inhibitors on Small Autosomal Values

4.2.1 IPC Values for Neat Blood

Neat liquid whole blood displayed higher IPC values than liquid whole blood diluted tenfold. Additionally, neat blood had lower amounts of small autosomal DNA (ng/

μL) compared to that of blood diluted tenfold after factoring out dilution. As stated previously, red blood cells contain inhibitors that prevent downstream PCR analysis. An IPC value that is high suggests that there are inhibitors present that are affecting the proper amplification of DNA. Thus, when blood is not diluted, more inhibitors are present, causing a higher IPC value with a smaller small autosomal value.

4.2.2 IPC Values for Direct Lysis Procedures

Samples extracted using Casework Direct diluted tenfold displayed the lowest IPC values and largest small autosomal values while samples extracted using Chelex® displayed the highest IPC values and lowest small autosomal values. One explanation for Casework Direct displaying the lowest IPC values and thus the highest small autosomal values is that lysates from samples that had been extracted using Casework Direct were diluted tenfold prior to qPCR analysis.

5. CONCLUSIONS

5.1 Conclusions from Data

Casework Direct performed the best overall compared to PicoPure®, Chai, and Chelex® and fabric type had no effect on DNA recovery from any of the direct lysis methods. Casework Direct recovered the highest amount of DNA after initial extraction and left the smallest amount of DNA behind on the substrates after re-extraction. However, it is important to note that Casework Direct extracts that are not diluted tenfold yielded data with "Undetermined" IPC values. This is an additional step to the workflow that must be considered when extracting blood using Casework Direct.

5.2 Considerations for Future Research

Future considerations can be applied concerning inhibitors left behind in the lysate after extraction of red blood cells. Recovering a large amount of DNA after the extraction is only relevant if a suitable STR profile can be generated afterward. Further studies should be conducted on Casework Direct at different dilutions to evaluate if a suitable STR profile can be made.

Additionally, IPC and small autosomal values extracted from stepwise blood dilutions using Casework Direct, PicoPure®, and Chai can be compared to understand the presence of inhibitors in blood on the reliability of the small autosomal values generated from qPCR.

APPENDIX:

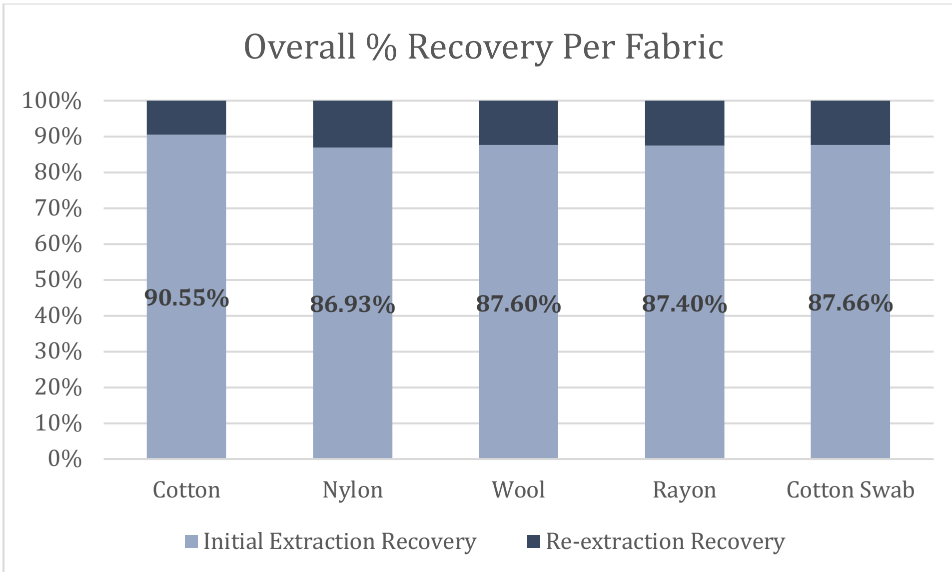

Figure A. Percentage of DNA recovered from each fabric.

LIST OF JOURNAL ABBREVIATIONS

Chem Biol Drug Des	Chemical Biology & Drug Design
Electron J Biotechnol	Electronic Journal of Biotechnology
Enzyme Microb Technol	Enzyme and Microbial Technology
Forensic Sci Int	Forensic Science International
Forensic Sci Int Genet	Forensic Science International Genetics
Forensic Sci Int Genet Suppl	Forensic Science International Genetics Supplement Series
Int Congr Ser	International Congress Series
J Biomater Sci Polym Ed	Journal of Biomaterials Science Polymer Edition
J Biomed Biotechnol	Journal of Biomedicine & Biotechnology
J Clin Microbiol	Journal of Clinical Microbiology
J Forensic Sci	Journal of Forensic Science
J Funct Biomater	Journal of Functional Biomaterials
J Legal Med	International Journal of Legal Medicine
Malays J Med Sci	The Malaysian Journal of Medical Sciences
Nat Commun	Nature Communications
Nat Rev Genet	Nature Review Genetics
Proc Natl Acad Sci	Proceedings of the National Academy of Sciences of the United States of America
Text Res J	Textile Research Journal
WIREs Forensic Sci	WIREs Forensic Science

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